*J. Eukaryot. Microbiol.*, 53(S1), 2006 pp. S77–S79

DOI: 10.1111/j.1550-7408.2006.00180.x

# Infectivity of Microsporidian Spores Exposed to Temperature Extremes and Chemical Disinfectants

**XUNDE LI[a] and RONALD FAYER[b]**

[a]*Veterinary Medical Teaching and Research Center, University of California-Davis, Tulare, California, and*
[b]*Environmental Microbial Safety Laboratory, Agricultural Research Service, United States Department of Agriculture, Beltsville, Maryland 20705*

MICROSPORIDIA in the genus *Encephalitozoon* are obligate intracellular parasites transmitted by spores via the fecal–oral route. *Encephalitozoon intestinalis*, *E. hellem*, and *E. cuniculi* infect humans and wild and domesticated animals. Relevant reservoirs and the routes of spread of these species infecting humans remain largely unknown. However, detection of spores in groundwater, water used for drinking, and in an outbreak thought to be waterborne strongly suggest the potential and importance of waterborne transmission (Cotte et al. 1999; Dowd, Gerba, and Pepper 1998; Fournier et al. 2000). Rendering spores non-infectious is extremely important for food and water protection and for safe facility management but data on inactivation and disinfection of spores are sparse. The present studies were designed to investigate the effects of ethanol, chlorine, heating and freezing on infectivity of *E. intestinalis*, *E. hellem*, and *E. cuniculi* spores using cell culture as a bioassay.

## MATERIALS AND METHODS

Spores of *E. intestinalis* originally isolated from an AIDS patient and propagated in cell culture (Didier et al. 1996) were provided by Elizabeth Didier, Tulane Regional Primate Center, Covington, Louisiana. Spores of *E. hellem* (ATCC No. 50451) and *E. cuniculi* (ATCC No. 50602) were purchased frozen in medium containing 10% DMSO cryoprotectant (American Type Culture Collection, Manassas, Virginia). All spores were propagated in mammalian cell cultures as described (Li et al. 2003). For each ethanol, chlorine, freezing, and heating treatment 150,000 spores of each species were placed in individual tubes and treated separately. Treated spores and untreated control spores were bioassayed for infectivity in MDBK cell monolayers grown in eight-well Lab-Tek chamber slides (Nalge Nunc Intl, Naperville, IL). Spores from every tube were inoculated into three wells (50,000 spores each) and cultured, fixed, stained, and examined by microscopy as described (Li et al. 2003). Intracellular clusters of developing microsporidia in each well were counted. For each treatment, three uninoculated cell monolayers served as negative controls.

**Ethanol treatment.** Six tubes per species each contained spores suspended in 1 ml of 70% ethanol in 1.5 ml microcentrifuge tubes. Duplicate tubes were prepared for each treatment at each of three time points while a seventh tube of spores suspended in Hanks balanced salt solution (HBSS) for 20 min served as an untreated positive control. Spore suspensions were incubated for 5, 10, and 20 min at 22 °C, washed three times in HBSS by centrifugation at 2,000 *g* for 6 min, then suspended in culture medium and inoculated into MDBK cell monolayers.

**Chlorine treatment.** Commercial laundry bleach (5.25% sodium hypochlorite) was diluted 1:50,000, 1:10,000 and 1:1,000 with deionized water (v:v). The pH value was measured with an ATI Orion pH meter (model 330, Orion, Boston, MA). Free available chlorine and total residual chlorine were measured using a digital chlorine colorimeter kit (model DC1100, LaMotte Co., Chestertown, MD). Spores of each species were placed in 13 microcentrifuge tubes and 1 ml of chlorine solution was added to 12 tubes (three dilutions × four time periods + one chlorine − free control). Spores were exposed at 22 °C for 10, 30, 60, and 120 min, washed three times with HBSS as above, suspended in culture medium, and inoculated into MDBK cell cultures.

**Freezing.** Spores of each species suspended in 1 ml of deionized water (pH 7.7) in microcentrifuge tubes were held at − 20 °C for 2, 4, 8, 12 and 24 h. Spores held at 4 °C for 24 h served as a positive control. Two tubes containing spores of each species were thawed to room temperature at each time point. These and controls were concentrated by centrifugation at 2,000 *g* for 6 min, suspended in culture medium and inoculated into MDBK cell monolayers.

**Heating.** Of six microtube PCR vials (no. T53-300, LabSource, Chicago, IL) containing spores of each species suspended in 500 µl DMEM culture medium, five vials were inserted into wells of a DNA Thermal Cycler (Perkin-Elmer, Cetus, Norwalk, CT) and held at 50, 60, 70, 80, and 100 °C, respectively, for 1 min (Table 1). In a second trial, three of four vials containing spores of each species were placed in the Thermal Cycler and held at 100 °C for 2, 5, and 10 min. For each trial one vial was held at 22 °C as an untreated control. Spores from each treated and control vial were then inoculated into MDBK cell monolayers (Table 1).

The effect of treatments was expressed as percent inhibition calculated as the [(mean number of intracellular clusters of microsporidia in wells inoculated with control spores) − (mean number of clusters in wells inoculated with treated spores) (mean number of clusters in wells inoculated with control spores)] × 100. Wells in which no clusters were found were recorded as 100% inhibition, although it is recognized that this figure reflects test limits and is not absolute.

Table **1.** Effect of exposure to elevated temperatures on infectivity of microsporidian spores for cultured cells.[a]

| Temperature vs Time (C/min) | *Encephalitozoon cuniculi* | *Encephalitozoon hellem* | *Encephalitozoon intestinalis* |
|---|---|---|---|
| 50/1 | 73.6 | 12.1 | 67.9 |
| 60/1 | 74.9 | 91.1 | 98.3 |
| 70/1 | 84.4 | 98.8 | 98.9 |
| 80/1 | 94.0 | 99.1 | 99.1 |
| 100/1 | 100 | 99.4 | 99.7 |
| 100/2 | 100 | 96.9 | 99.9 |
| 100/5 | ND | 99.7 | 100 |
| 100/10 | ND | 100 | 100 |

[a]Results are expressed as percentage inhibition of microsporidia growth.
ND, not determined.

Corresponding Author: R. Fayer, Environmental Microbial Safety Laboratory, Agricultural Research Service, United States Department of Agriculture, Bldg. 173 Powder Mill Road, Beltsville, Maryland 20705, USA—Telephone number: +-301-504-8750; FAX number: +-301-504-6608; e-mail: rfayer@anri.barc.usda.gov

Table **2.** Effects of exposure to chlorine on infectivity of microsporidian spores for cultured cells.

| Dilution Factor | Chlorine level[b] | pH | Percent inhibition of microsporidia growth[a] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *Encephalitozoon cuniculi* | | | | *Encephalitozoon hellem* | | | | *Encephalitozoon intestinalis* | | | |
| | | | Exposure time (min)[a] | | | | | | | | | | | |
| | | | 10 | 30 | 60 | 120 | 10 | 30 | 60 | 120 | 10 | 30 | 60 | 120 |
| 1:50,000 | 0.54/0.66 | 5.2 | 99.1[b] | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 98.2 | 98.6 | 98.9 | 99.2 |
| 1:10,000 | 2.55/2.87 | 5.0 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 99.2 | 99.3 | 99.3 | 99.8 |
| 1:1,000 | 25.7/31.3 | 4.4 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |

[a]Results are expressed as percentage of inhibition on parasite development in a bioassay as described in ''Materials and Methods.''
[b]Free available chlorine/total residual chlorine expressed in mg/L.

## RESULTS AND DISCUSSION

Spores of *E. intestinalis* and *E. cuniculi* exposed to 70% ethanol for 5 min and those of *E. hellem* exposed for 20 min were rendered 100% non-infectious for MDBK cell monolayers. Similar results were obtained in studies by others. Exposure to 70% ethanol for 30 min (Waller 1979) or even 10 min (Shadduck and Polley 1978) entirely inhibited *E. cuniculi* growth in cell culture.

The free available chlorine/total remaining chlorine at dilutions of 1:50,000, 1:10,000 and 1:1,000 laundry bleach:water, respective pH values, and percent inhibition of growth after exposure of spores to chlorine are shown in Table 2. After *E. hellem* spores were exposed to laundry bleach diluted 1:50,000 (0.54 mg chlorine/L) for 10 min, growth in MDCK cells was inhibited 100%. Spores of *E. cuniculi* had to be exposed at the same dilution for 30 min or in laundry bleach diluted 1:10,000 (2.55 mg chlorine/L) for 10 min before they were 100% inhibited. Although *E. intestinalis* spores exposed to laundry bleach diluted 1:50,000 for 10 min resulted in >98% inhibition, exposure at a dilution of 1:1,000 (25.7 mg chlorine/L) for 10 min was required to obtain 100% inhibition. In an earlier study, *E. intestinalis* spores exposed to chlorine at 2 mg/L for 16 min were inhibited 99.9% in a rabbit kidney cell culture bioassay, suggesting this concentration was effective for water treatment (Wolk et al. 2000). Microsporidian spores from household water tanks, exposed overnight to chlorine concentrations of 4 and 8 mg/L, were completely non-infective for animals (Khalifa, El Temsahy, and Abou El Naga 2001). However, in that report the species of microsporidia was not identified and there was no indication that positive control specimens were tested to determine if animals were susceptible to infection from untreated spores. The U.S. Environmental Protection Agency has recently revised the maximum concentration of chlorine allowed in drinking water at treatment plants to 4 mg/L. Data from the present study found that exposure of *E. cuniculi* and *E. hellem* spores to a chlorine level of 2.55 mg/L for 10 min was sufficient to render 100% of the spores non-infective and to render 99.8% of the spores of *E. intestinalis* non-infective after exposure for 120 min. Because spores in the present study were exposed at 22 °C, the lower temperatures of chlorinated drinking water may require longer periods of exposure to reduce infectivity of spores to levels obtained under experimental conditions but higher concentrations of chlorine might compensate for lower temperatures. Conventional levels of chlorine (1–3 mg/L) used in swimming pools where water temperatures normally reach or exceed 22 °C should be adequate to greatly reduce or eliminate the infectivity of *E. intestinalis, E. hellem* and *E. cuniculi* spores after relatively short exposure times.

After *E. intestinalis* and *E. hellem* spores in water without cryoprotectants were held at −20 °C for 24 h, and *E. cuniculi* spores were held at the same temperature for only 2 h, growth in cell culture was inhibited 100%. Results obtained in studies with other species or isolates of microsporidia and with other methods vary widely. Infectivity of *Nosema apis* spores in bees held at −20 °C for 24 h differed little from fresh spores (Bailey 1972). Spores from 31 species of microsporidia in water held in liquid nitrogen for 2–25 yr were infective for insect hosts (Maddox and Solter 1996). Spores of *Glugea stephani*, from flatfish, held at −19 °C for 24 h were considered viable based on a filament extrusion test (Amigo et al. 1996). In contrast, spores of *Loma salmonae* in DMSO, Earle's balanced salt solution, or water held at −20 °C and −70 °C for 24 h did not infect fish (Shaw, Kent, and Adamson 2000). Spores of *E. cuniculi* in water at 4 °C for 2 yr or at −12 °C and −24 °C for 1, 8, and 24 h were infective for SCID mice; those held at −70 °C for 1 and 8 h were much less infective (Koudela, Kucerova, and Hudcovic 1999). In contrast, another study reported that spores of *E. cuniculi* in medium 199 held 1 days at −20 °C and those held at 4 °C for 98 days were not infective (Waller 1979). Still others reported that *E. cuniculi* spores, in medium 199 with DMSO and glycerol, held at −70 °C or in liquid nitrogen for 6 mo infected rabbit choroid plexus cell cultures (Shadduck and Polley 1978). Some *E. hellem* spores in water frozen four times at −15 °C for unknown times infected green monkey kidney cell cultures (Kucerova-Pospisilova et al. 1999). Differences in species, genotypes, and assay methods likely account for the diversity of findings in the foregoing reports.

Spores of *E. cuniculi*, *E. intestinalis* and *E. hellem* were exposed to temperatures of 50 °C–100 °C with higher rates of inhibition correlated to increasing temperature and time of exposure (Table 1). Spores of *E. cuniculi* held at 100 °C for 1 min failed to grow in cell culture whereas spores of *E. intestinalis* and *E. hellem* had to be held for 5 and 10 min, respectively, to obtain 100% inhibition of growth. Others have observed similar effects of exposure of *E. cuniculi* spores to elevated temperatures. Spores held at 60 °C for 1 min but not those held for 5 min were infective for mice (Koudela et al. 1999). Spores in HBSS held at 56 °C for 60 min but not those held at 120 min were infective for cells (Shadduck and Polley 1978). Only 2.5% of spores held at 56 °C for 30 min survived but boiling for 5 min or autoclaving at 120 °C for 10 min killed all spores (Waller 1979). Collectively the present findings and those of others demonstrate that spores of *Encephalitozoon* species differ in their ability to survive exposure to elevated temperatures but exposure to 100 °C for 10 min renders non-infective all three species of public health concern.

## ACKNOWLEDGMENTS

The authors gratefully acknowledge the technical contributions of Robert Palmer.

## LITERATURE CITED


Amigo, J. M., Gracia, M. P., Rius, M., Salvado, H., Maillo, P. A. & Vivares, C. P. 1996. Longevity and effects of temperature on the viability and polar-tube extrusion of spores of *Glugea stephani,* a microsporidian parasite of commercial flatfish. *Parasitol. Res.*, **82**:211–214.

Bailey, L. 1972. The preservation of infective microsporidian spores. *J. Invert. Pathol.*, **20**:252–254.

Cotte, L., Rabodonirina, M., Chapuis, F., Bailly, F., Bissuel, F., Raynal, C., Gelas, P., Persat, F., Piens, M. A. & Trepo, C. 1999. Waterborne outbreak of intestinal microsporidiosis in persons with and without human immunodeficiency virus infection. *J. Infect. Dis.*, **180**:2003–2008.

Didier, E. S., Rogers, L. B., Orenstein, J. M., Baker, M. D., Vossbrinck, C. R., van Gool, T., Hartskeerl, R., Soave, R. & Beaudet, L. M. 1996. Characterization of *Encephalitozoon (Septata) intestinalis* isolates cultured from nasal mucosa and bronchoalveolar lavage fluids of two AIDS patients. *J. Eukaryot. Microbiol.*, **43**:34–43.

Dowd, S., Gerba, C. & Pepper, I. 1998. Confirmation of the human pathogenic microsporidia *Enterocytozoon bieneusi, Encephalitozoon intestinalis,* and *Vittaforma corneae* in water. *Appl. Environ. Microbiol.*, **64**:3332–3335.

Fournier, S., Liguory, O., Santillana-Hayat, M., Guillot, E., Sarfati, C., Dumoutier, N., Molina, J. & Derouin, F. 2000. Detection of microsporidia in surface water: a one-year follow-up study. *FEMS Immun. Med. Microbiol.*, **29**:95–100.

Khalifa, A. M., El Temsahy, M. M. & Abou El Naga, I. F. 2001. Effect of ozone on the viability of some protozoa in drinking water. *J. Egypt. Soc. Parasitol.*, **31**:603–616.

Koudela, B., Kucerova, S. & Hudcovic, T. 1999. Effect of low and high temperature on infectivity of *Encephalitozoon cuniculi* spores suspended in water. *Folia Parasitol. (Praha)*, **46**:171–174.

Kucerova-Pospisilova, Z., Carr, D., Leitch, G., Scanlon, M. & Visvesvara, G. S. 1999. Environmental resistance of *Encephalitozoon* spores. *J. Eukaryot. Microbiol.*, **46**:11S–13S.

Li, X., Palmer, R., Trout, J. M. & Fayer, R. 2003. Infectivity of microsporidia spores stored in water at environmental temperatures. *J. Parasitol.*, **89**:185–188.

Maddox, J. V. & Solter, L. F. 1996. Long-term storage of infective microsporidian spores in liquid nitrogen. *J. Eukaryot. Microbiol.*, **43**:221–225.

Shadduck, J. A. & Polley, M. B. 1978. Some factors influence the in vitro infectivity and replication of *Encephalitozoon cuniculi*. *J. Protozool.*, **25**:491–496.

Shaw, R. W., Kent, M. L. & Adamson, M. L. 2000. Viability of *Loma salmonae* (Microsporidia) under laboratory conditions. *Parasitol. Res.*, **86**:978–981.

Waller, T. 1979. Sensitivity of *Encephalitozoon cuniculi* to various temperature, disinfectants and drugs. *Lab. Anim.*, **13**:227–230.

Wolk, D. M., Johnson, C. H., Rice, E. W., Marshall, M. M., Grahn, K. F., Plummer, C. B. & Sterling, C. R. 2000. A spore counting method and cell culture model for chlorine disinfection studies of *Encephalitozoon* syn. Septata intestinalis.. *Appl. Environ. Microbiol.*, **66**:1266–1273.